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DECREASED PLATELET INHIBITION BY NITRIC OXIDE IN TWO BROTHERS  
WITH A HISTORY OF ARTERIAL THROMBOSIS

BY

J.E. FREEDMAN, J. LOSCALZO, S.E. BENOIT, C.R. VALERI,  
M.R. BARNARD, AND A.D. MICHELSON

NAVAL BLOOD RESEARCH LABORATORY  
BOSTON UNIVERSITY SCHOOL OF MEDICINE  
615 ALBANY STREET  
BOSTON, MA 02118

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## ABSTRACT

Highly reactive oxygen species rapidly inactivate nitric oxide (NO), an endothelial product which inhibits platelet activation. We studied platelet inhibition by NO in two brothers with a cerebral thrombotic disorder. Both children had hyperreactive platelets, as determined by whole blood platelet aggregometry and flow cytometric analysis of the platelet surface expression of P-selectin. Mixing experiments showed that the patients' platelets behaved normally in control plasma; however, control platelets suspended in patient plasma were not inhibited by NO. As determined by flow cytometry, in the presence of plasma from either patient there was normal inhibition of the thrombin-induced expression of platelet surface P-selectin by prostacyclin, but not NO. Using a scopoletin assay, we measured a 2.7-fold increase in plasma  $H_2O_2$  generation in one patient and a 3.4-fold increase in the second patient, both compared with control plasma. Glutathione peroxidase (GSH-Px) activity was decreased in the patients' plasmas compared with control plasma. The addition of exogenous GSH-Px led to restoration of platelet inhibition by NO. These data show that, in these patients' plasmas, impaired metabolism of reactive oxygen species reduces the bioavailability of NO and impairs normal platelet inhibitory mechanisms. These findings suggest that attenuated NO-mediated platelet inhibition produced by increased reactive oxygen species or impaired antioxidant defense may cause a thrombotic disorder in humans.

Key words: child, cerebral embolism and thrombosis, blood platelet, nitric oxide, glutathione peroxidase

## INTRODUCTION

An important antithrombotic product of the endothelial cell is endothelium-derived relaxing factor (EDRF), which inhibits platelet aggregation<sup>1,2</sup> and prevents adhesion of platelets to the endothelium<sup>3</sup>. EDRF, or endothelium-derived nitric oxide (NO),<sup>4,5</sup> mediates its effects by elevating intracellular levels of cyclic 3',5'-guanosine monophosphate (cGMP). These alterations in cGMP levels are believed to represent a negative feedback pathway in the regulation of platelet activation. NO has been recently shown to inhibit the normal activation-dependent increase in the expression of platelet surface glycoproteins, including P-selectin and the integrin glycoprotein IIb-IIIa complex.<sup>6</sup> Endogenously synthesized NO has been shown to prevent thrombosis in a model of endotoxin-induced glomerular damage,<sup>7</sup> as well as attenuate platelet adhesion to damaged endothelium<sup>8</sup>.

NO is known to interact with several reactive oxygen species, such as superoxide ( $O_2^{\cdot-}$ ), to form peroxynitrite.<sup>9,10</sup> This potent oxidant can lead to lipid peroxidation and lipid radical chain propagation reactions.<sup>11</sup> NO has been shown to react with  $H_2O_2$ , producing a highly cytotoxic species believed to be singlet oxygen.<sup>12</sup> Such interactions may be important in the mechanism(s) of toxicity of reactive oxygen species, which includes cell and tissue damage secondary to oxidation of proteins, deoxyribonucleic acids, and lipids.

Oxidative reactions involving NO in plasma could modify the effect of NO on platelet function and, subsequently, induce thrombosis. While a recent study demonstrated that systemic inhibition of EDRF production decreased bleeding time in humans<sup>13</sup>, alterations in NO production or metabolism have not yet been shown to be pathophysiologically relevant *in vivo*.

In this study, we examined the plasma and platelets from two children with a history of thrombosis and found an attenuation of the normal platelet inhibitory response to NO as a result of impaired metabolism of reactive oxygen species. These observations represent the first report of a thrombotic diathesis caused by a functional insufficiency of nitric oxide, and highlight the importance of endothelial nitric oxide in modulating platelet function *in vivo*.

## Case Report

Patient 1 (date of birth 11/8/85) was a generally well white boy who presented at 13 months of age with right-sided hemiparesis, right facial palsy, and slurred speech. An arteriogram of his left carotid and intracranial arteries performed three days later was completely unremarkable, without evidence of vasculitides, arteriovenous malformations, or Moyamoya disease. The hemiparesis and slurred speech completely resolved within one week. Cerebrospinal fluid cell count and protein, glucose, pyruvate, and lactate levels were normal. Chest X-ray, electrocardiogram (EKG), echocardiogram, Holter monitor, electroencephalogram (EEG) with photic drive, serum pyruvate and lactate, and muscle biopsy were all normal. Computed tomography (CT) scan of the head performed one month later showed a small infarct of the left basal ganglion. At 22 months of age, patient 1 had a 24-hour period of intermittent left-sided hemiparesis, left facial palsy, and slurred speech, after which there was complete resolution of these signs. An arteriogram of the right carotid and intracranial arteries was normal. Chest Xray, EKG, echocardiogram, and serum pyruvate and lactate were all again normal, as were cerebrospinal fluid cell count and protein, glucose, pyruvate, and lactate levels. Again, a follow-up CT scan showed a small infarct of the basal ganglion, this time on the right side. His final clinical diagnosis was, therefore, considered to be two separate episodes of cerebral arterial thrombosis with infarction.

Patient 2 (date of birth 7/16/90) is the brother of patient 1. Patient 2 had been generally well until he presented at 15 months of age with a one-month history of intermittent ataxia and right-sided hemiparesis. Magnetic resonance imaging (MRI) of the head was normal. His neurological examination rapidly returned to normal. His final clinical diagnosis was transient ischemic attacks.

There was no history of head trauma or dehydration in either brother. Prior to the thrombotic episodes, neither child was taking any medications. Developmental milestones were

normal in both brothers before and after the thrombotic episodes. Neither brother has had hypertension. There is no family history of thromboembolism. The mother (date of birth 6/26/52) has systemic lupus erythematosus without renal or central nervous system involvement. The patients' sister (date of birth 4/13/87) has no history of thrombotic disease or vasculitis.

The following tests were normal in both boys: plasma antithrombin III level, plasma protein C and S, prothrombin time, partial thromboplastin time, complete blood count, blood urea nitrogen, serum creatinine, serum sodium, serum potassium, serum chloride, serum calcium, serum magnesium, blood glucose level, liver function tests, serum cholesterol, and serum HDL. The following tests were negative in both boys: urinary and serum homocysteine, lupus inhibitor, anticardiolipin antibody, antinuclear antibody, rheumatoid factor, and sickle cell screen. The two brothers are currently being treated with aspirin and have not experienced any further thrombotic episodes.

Further laboratory investigations described in this paper were performed between 1991 and 1995. All assays were performed more than three months after the cerebrovascular thrombotic events to avoid the transient platelet hyperreactivity reported to occur within the first six weeks after a stroke.<sup>14</sup>

## MATERIALS AND METHODS

### Chemicals and Solutions

H<sub>2</sub>O<sub>2</sub>, glutathione reductase (baker's yeast), glutathione peroxidase (GSH-Px, human or bovine erythrocyte), superoxide dismutase (SOD), reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH), D,L-homocysteine, L-cysteine, N-acetyl-L-cysteine, sodium nitrite, bovine serum albumin (BSA), hydrochloric acid, perchloric acid, trichloroacetic acid, Sepharose 2B, adenosine 5'-diphosphate (ADP), thrombin, arachidonic acid, collagen, scopoletin, horseradish peroxidase, and glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO). Tyrode's-Hepes-buffered saline consisted of 140 mM NaCl, 6 mM *N*-[2-hydroxyethyl]-piperazine-*N*-[2-ethane-sulfonic acid], 2 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% dextrose, pH 7.4, and 0.4% BSA. Krebs's buffer consisted of 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 12.5 mM NaHCO<sub>3</sub>, pH 7.4, and 11 mM D-glucose. Cyclic GMP enzyme immunoassay kits were purchased from Cayman Chemical Co. (Ann Arbor, MI).

### EDRF/NO

The EDRF congeners S-nitroso-N-acetylcysteine (SNAC) and S-nitrosoglutathione (SNO-Glu) were prepared by reacting freshly prepared solutions of *N*-acetylcysteine or GSH with NaNO<sub>2</sub> at acidic pH, as previously described.<sup>15,16</sup> SNAC and SNO-Glu were prepared within 10 minutes of use, kept at 4°C, and diluted as necessary into aqueous buffer immediately before addition to assay systems.

### Monoclonal Antibodies

#### *P-selectin-specific:*

S12 (provided by Dr. Rodger P. McEver, University of Oklahoma) is a murine monoclonal antibody directed against P-selectin.<sup>17,18</sup> P-selectin, also referred to as CD62P,<sup>19</sup> GMP-140,<sup>17</sup> and PADGEM protein,<sup>20</sup> is a component of the  $\alpha$ -granule membrane of resting platelets that is only expressed on the platelet surface membrane after platelet degranulation and secretion.<sup>17,21</sup>

*Glycoprotein IIb-IIIa-specific:*

Y2/51 (Dako Corporation, Carpinteria, CA) is a murine monoclonal antibody directed against platelet membrane glycoprotein IIIa (CD41),<sup>22</sup> while PAC1 (provided by Dr. Sanford J. Shattil, University of Pennsylvania, Philadelphia) is a murine monoclonal antibody directed against the fibrinogen binding site exposed by a conformational change in the glycoprotein IIb-IIIa complex (CD41/61) of activated platelets.<sup>23</sup> Unlike Y2/51,<sup>24</sup> PAC1 does not bind to resting platelets.<sup>23</sup> Although Y2/51 and PAC1 were used in the same assays, these antibodies do not interfere with the binding of one another to activated platelets (data not shown).

*Glycoprotein Ib-IX-specific:*

6D1 (provided by Dr. Barry S. Coller, Mount Sinai Medical Center, New York) is a murine monoclonal antibody directed against the von Willebrand factor binding site on the aminoterminal domain of platelet membrane glycoprotein Ib $\alpha$  (CD42b).<sup>25,26</sup>

S12, PAC1, and 6D1 were biotinylated, as previously described.<sup>27,28</sup> Fluorescein isothiocyanate (FITC)-conjugated Y2/51 was purchased from Dako.

**Preparation of Platelet-rich Plasma (PRP)**

Peripheral blood was drawn from healthy adult volunteers who had not consumed acetylsalicylic acid or any other platelet inhibitor for at least seven days. The first 2 mL of blood drawn were discarded. Blood was then drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ), which does not result in platelet activation.<sup>24</sup> The citrated blood was centrifuged (150 x g, 15 minutes, 22°C) and the supernatant (PRP) was separated. The sample was then diluted 1:4 in modified Hepes-Tyrod's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM MgCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose), pH 7.4. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP or venous blood at 1200xg for 10 minutes.

### **Preparation of Whole Blood Samples for Flow Cytometry**

Samples were prepared as previously described.<sup>24,27</sup> Blood was drawn by venipuncture into a Vacutainer, as described above. Within 15 minutes of drawing, the blood was diluted 1:4 in modified Hepes-Tyrod's buffer, pH 7.4.

### **Preparation of Washed Platelets**

Washed platelets were prepared as previously described.<sup>29,30</sup> PRP was prepared as described above. After addition to the PRP of citrate albumin wash buffer (128 mM NaCl, 4.3 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 7.5 mM  $\text{Na}_2\text{HPO}_4$ , 4.8 mM sodium citrate, 2.4 mM citric acid, 0.35% bovine serum albumin, 11 mM glucose), pH 6.5, with 50 ng/ml prostaglandin (PG)  $\text{E}_1$ , washed platelets were prepared by centrifugation. The concentration of washed platelets was adjusted to 150,000/ $\mu\text{L}$  in modified Hepes-Tyrod's buffer, pH 7.4.

### **Preparation of Gel-Filtered Platelets**

Gel-filtered platelets (GFP) were obtained by passing PRP over a Sepharose-2B column in Tyrod's-Hepes buffered saline, as previously described.<sup>31</sup> Platelet counts were determined using a Coulter Counter, model ZM (Coulter Electronics, Hialeah, FL). Platelets were adjusted to  $1.5 \times 10^8$  platelets/ml by the addition of PPP or Tyrod's-Hepes-buffered saline.

### **Incubations for Flow Cytometry**

Washed platelets (75,000/ $\mu\text{L}$ ), diluted PRP, or diluted whole blood was incubated at 22°C for 10 minutes with: a) either 10  $\mu\text{M}$  SNAC, 10  $\mu\text{M}$  prostacyclin (Sigma), or buffer only; and, b) either 0.25  $\mu\text{M}$  of the stable thromboxane  $\text{A}_2$  analog U46619 (Cayman Chemical, Ann Arbor, MI), 0.05 - 1 U/mL of purified human  $\alpha$ -thrombin (provided by Dr. John W. Fenton II, New York Department of Health, Albany), or buffer only. In PRP and whole blood samples incubated with thrombin, 2.5 mM of the peptide L-glycyl-L-prolyl-L-arginyl-L-proline (GPRP) (Calbiochem, San Diego, CA) was included to inhibit fibrin polymerization.<sup>27,32</sup> Samples were then fixed in formaldehyde (1% final concentration) for 30 minutes at 22°C and diluted 10-fold in modified Tyrod's buffer, pH 7.4. The samples were then incubated (22°C, 20 minutes) with a



near saturating concentration of FITC-conjugated monoclonal antibody Y2/51 (glycoprotein IIIa-specific) and a saturating concentration of a biotinylated monoclonal antibody (either S12 or 6D1), followed by an incubation (22°C, 20 minutes) with 30 µg/mL phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA). Monoclonal antibody PAC1 does not bind well to fixed platelets. For this reason, as distinct from the other biotinylated antibodies used in this study, PAC1 was incubated with platelets before fixation, as previously described.<sup>24,28</sup> All samples were then diluted 10-fold in modified Tyrode's buffer, pH 7.4, and stored at 4°C until flow cytometric analysis was performed (within 24 hours). This method results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody tagging.<sup>24</sup>

### **Flow Cytometry**

Samples of washed platelets, PRP, and whole blood were analyzed in an EPICS Profile II flow cytometer (Coulter Cytometry, Hialeah, FL) as previously described.<sup>27,29,30</sup> The flow cytometer was equipped with a 500 mW argon laser (Cyronics, San Jose, CA) operated at 15 mW and a wavelength of 488 nm. The fluorescence of FITC and phycoerythrin were detected using 525 nm and 575 nm band pass filters, respectively. After identification of platelets by gating on both Y2/51-FITC positivity (i.e. glycoprotein IIIa-positivity) and their characteristic light scatter, binding of the biotinylated monoclonal antibody (S12, PAC1, or 6D1) was determined by analyzing 5,000 individual platelets for phycoerythrin fluorescence. Background binding, obtained from parallel samples run with FITC-Y2/51 and purified biotinylated mouse IgM (for PAC1 assays) or IgG (for all other assays) (Boehringer Mannheim, Indianapolis, IN), was subtracted from each test sample.

### **Platelet Aggregation**

Platelet aggregation in whole blood was measured at 37°C using a whole blood aggregometer (Chrono-Log Corp., Havertown, PA). Whole blood anticoagulated with sodium

citrate was treated with thrombin, arachidonic acid, or collagen, and the aggregation patterns were measured and analyzed by digitizing the area under the curve over a five-minute period.

Aggregation of GFP and PPP was monitored using a standard nephelometric technique in which changes in light transmittance are recorded as a function of time.<sup>33</sup> Platelet aggregation experiments were initially conducted using 0.2 ml GFP from the different family members mixed with 0.2 ml of PPP from a healthy donor. Aggregations were induced by adding 5  $\mu$ M ADP and the absolute extent of change of light transmittance was recorded in a four-chamber aggregometer (BioData, Hatboro, PA). In other experiments, PPP from each of the family members was mixed with GFP from a healthy donor. GFP and PPP were also incubated for one minute at 37°C with an antioxidant enzyme, S-nitroso-glutathione (SNO-Glu), or an antioxidant enzyme and SNO-Glu prior to the addition of ADP.

#### **Cyclic Nucleotide Assay**

Trichloroacetic acid (final concentration, 5% vol/vol) was added to PRP. Samples were vortexed, placed on ice, and centrifuged at 1500xg for 10 minutes at 4°C. The supernatant was extracted with diethyl ether and assayed for cGMP by an enzyme-linked immunoadsorbent assay (ELISA) methodology using cGMP antiserum (Cayman Chemical Co., Ann Arbor, MI).

#### **Measurement of NO and S-Nitrosothiols**

The production of NO was measured by the use of a photolysis-chemiluminescence system (Thermedics, Inc. Woburn, MA) as previously described.<sup>34</sup> The system detects free NO or NO photolytically cleaved from a thionitrite carrier adduct. The solution or extract containing NO and its adducts is injected into a borosilicate glass coil through the center of which is a mercury arc lamp emitting light of 300-400 nm, which photolytically cleaves the S-NO bond. The free NO is separated from the solvent and thiyl radicals with cold traps, and carried by helium into the reaction chamber for chemiluminescence detection.<sup>35</sup> SNO-Glu was used to derive a standard curve, the correlation coefficient for which was typically 0.99.

### **Hydrogen Peroxide Generation**

The quantity of peroxide in solution was determined by measuring the extinction of scopoletin fluorescence during oxidation of the fluorophor by horseradish peroxidase.<sup>36</sup> Assay conditions detected H<sub>2</sub>O<sub>2</sub> generated in solution by homocysteine, according to published methods.<sup>37,38</sup> Reactions were performed in cuvettes containing 4  $\mu$ M scopoletin, 500  $\mu$ M homocysteine, and 4% PPP in Krebs's buffer. The reaction was initiated by the addition of 2.2  $\mu$ M horseradish peroxidase. Fluorescence measurements were performed using a spectrofluorimeter (Fluorolog 2 model F11; Spex Industries, Inc., Edison, NJ) with sample excitation at 360 nm and emission at 460 nm.

### **Determination of Glutathione Peroxidase Activity**

Endogenous plasma GSH-Px was assayed by coupling the peroxidase reaction with the reduction of oxidized glutathione (GSSG) by glutathione reductase using NADPH. Hydroperoxide reduction was followed by the decrease in absorbance of NADPH at 340 nm.<sup>39</sup> Activity was evaluated using GSH as the cosubstrate for GSH-Px.

### **Determination of Plasma Antioxidant Levels**

Selenium levels were determined by Dr. Georg Alfthan (National Public Health Institute, Helsinki, Finland) by electrothermal atomic absorption spectrometry as previously described.<sup>40</sup> Plasma  $\alpha$ -tocopherol content was determined using HPLC with electrochemical detection<sup>41</sup> and ascorbate and urate levels were measured by reversed-phase HPLC coupled with electrochemical detection<sup>42</sup> by Dr. Balz Frei (Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA). The low-molecular-weight thiols glutathione, cysteine, and homocysteine were measured by HPLC using a C18 reverse-phase column coupled to an electrochemical detector as previously described.<sup>43</sup>

## Statistics

All data are presented as the mean  $\pm$  standard error of the mean. Paired samples were compared by Student's  $t$  test; values of  $p < 0.05$  were considered significant. Groups of data were tested by analysis of variance and, if significant, were further evaluated by the Newman-Keuls test.

## RESULTS

### Platelet Reactivity as Determined by Whole Blood Platelet Aggregometry and Flow Cytometry

To determine whether the brothers' platelets were hyperreactive, studies were performed in whole blood in order to circumvent the possibility of artifactual *in vitro* platelet activation. Two whole blood methods were used: whole blood platelet aggregometry and whole blood flow cytometry. Compared to normal controls, the platelets of patients 1 and 2 were hyperreactive in response to thrombin, arachidonic acid, and collagen, as determined by whole blood aggregometry (Table 1). The platelets of patient 1 and, to a lesser extent, patient 2 were hyperreactive to U46619 (a stable thromboxane A<sub>2</sub> analogue), as determined by whole blood flow cytometric detection of i) increased platelet surface expression of P-selectin (reflecting  $\alpha$ -granule secretion, reported by monoclonal antibody S12); ii) increased exposure of the fibrinogen binding site on the glycoprotein IIb-IIIa complex (reported by monoclonal antibody PAC1); and iii) to a lesser extent, decreased platelet surface expression of glycoprotein Ib (the von Willebrand factor receptor, reported by monoclonal antibody 6D1) (Fig. 1). The increased platelet surface expression of P-selectin and the glycoprotein IIb-IIIa complex in patients 1 and 2 was not due to additional stores of these antigens, because maximal platelet activation with 10 U/ml thrombin resulted in similar surface exposure of P-selectin and the glycoprotein IIb-IIIa complex on the platelets of patients 1 and 2 and normal controls (data not shown). Circulating platelets were not significantly activated, as determined by lack of platelet surface P-selectin expression and the lack of fibrinogen binding sites on the glycoprotein IIb-IIIa complex of unstimulated whole blood samples (Fig. 1).

### Effect of SNAC and Prostacyclin on Platelet Surface Expression of P-selectin

Because platelet hyperaggregability and hyperreactivity were observed in patients 1 and 2 irrespective of whether they were or were not medicated with aspirin (80 mg per day) (data not shown), the possibility that EDRF/NO was involved in the mechanism was examined. S-nitroso-

N-acetylcysteine (SNAC), a biologically active NO donor,<sup>34</sup> inhibited thrombin-induced exposure of P-selectin on the surface of normal platelets in normal plasma (Fig. 2, top panel). Plasma from patient 1 or patient 2, but not control plasma, blocked this effect (Fig. 2). Studies with PRP from patients 1 and 2 demonstrated that SNAC did not inhibit the thrombin-induced exposure of platelet surface P-selectin (data not shown). Studies with washed platelets from patients 1 and 2 demonstrated that SNAC normally inhibited the thrombin-induced exposure of platelet surface P-selectin (data not shown). Taken together, these data demonstrate that the plasma, but not the platelets, of patients 1 and 2 blocked the inhibitory effect of SNAC on thrombin-induced platelet degranulation. Similar results were obtained when experiments were performed with U46619 rather than thrombin (data not shown). To determine intraassay variability, plasma from normal donors (n=10) was incubated with gel-filtered platelets. Incubation of platelets with thrombin 1 U/ml in the presence of 10  $\mu$ M SNAC resulted in an  $85.8 \pm 2.8\%$  (mean  $\pm$  SEM, n=10) reduction in platelet surface P-selectin compared with matched samples incubated with thrombin 1 U/ml in the absence of SNAC. The lack of any reduction in platelet surface P-selectin in the presence of plasma from patients 1 and 2 (Figure 2, middle and lower panels) is therefore very significant.

The effect of plasma from patients 1 and 2 was specific for NO because prostacyclin (another endothelium-derived inhibitor of platelet function<sup>44</sup> that may be synergistic with the platelet inhibitory effects of EDRF/NO<sup>45,46</sup>) blocked the thrombin-induced exposure of platelet surface P-selectin equally well in the whole blood of patients 1 and 2 as in the whole blood of normal donors (data not shown).

#### **Effect of SNO-Glu on Platelet Aggregation**

GFP from family members were mixed with PPP from a normal control, incubated with the NO donor SNO-Glu (5 $\mu$ M) for one minute, and aggregation induced with ADP (5 $\mu$ M). Normally, significant attenuation of platelet aggregation is observed after the addition of NO or S-nitrosothiols under these conditions. In the presence of PPP from a normal control, platelets

from the patients and family members were inhibited by SNO-Glu (data not shown). Next, GFP from a normal adult donor were mixed with PPP from family members in the presence or absence of SNO-Glu (5 $\mu$ M). Compared with the father, platelets in the presence of PPP from patient 2 failed to be inhibited by SNO-Glu (Fig. 3a). For incubations containing PPP from the father, sister, or pooled pediatric control, platelet aggregation was significantly inhibited in the presence of SNO-Glu (Fig. 3b). In the presence of PPP from patient 1, patient 2, or their mother, platelets failed to be inhibited by SNO-Glu.

#### **Plasma Concentration of Nitric Oxide and S-Nitrosothiols**

Protein-precipitated plasma from family members or controls was directly injected into a photolysis-chemiluminescence system to measure the level of total NO, free NO and low-molecular-weight S-nitrosothiols (Fig. 4). Compared to plasma from a pooled pediatric control, levels of NO in patients 1 and 2 were found to be significantly depressed (15% and 28%, respectively, compared to control). Plasma NO levels from both the mother and the unaffected sibling were also found to be significantly decreased as compared to the father and the pooled pediatric control plasma.

#### **Effect of SNO-Glu and PPP on Platelet cGMP**

PRP from family members or normal controls was assayed for platelet cGMP content, which has been shown to correlate with total plasma NO levels and to reflect the adequacy of platelet inhibition by endogenous EDRF/NO.<sup>47</sup> The platelet proteins were precipitated with trichloroacetic acid and the protein-free supernatant was analyzed for cGMP. Platelet cGMP levels for patients 1 and 2 were significantly decreased compared to pooled pediatric control platelets (Fig. 5).

#### **Effect of Plasma on H<sub>2</sub>O<sub>2</sub> Generation**

Hydrogen peroxide was determined by measuring the extinction of scopoletin fluorescence during its oxidation by horseradish peroxidase. The generation of H<sub>2</sub>O<sub>2</sub> by homocysteine was followed over a five-minute period in the presence of plasma (4 % vol/vol)

from family members or from a pooled pediatric control (Fig. 6). Plasma from patients 1, 2, or their mother led to sustained  $\text{H}_2\text{O}_2$  generation compared to the normal pooled pediatric control (2.7-, 3.4-, and 2.6-fold increase for patients 1, 2, and their mother, respectively). In the presence of plasma from the patients' father or clinically normal sibling, no significant  $\text{H}_2\text{O}_2$  formation was detected (Fig. 6). The formation of  $\text{H}_2\text{O}_2$  was also measured individually in normal plasma donors ( $n=8$ ). A mean change of -0.32 absolute units was detected over five minutes with a  $\text{SEM}=0.05$ , confirming the significance of the increased  $\text{H}_2\text{O}_2$  formation in the brothers and their mother.

### **Plasma Glutathione Peroxidase Activity**

The level of GSH-Px activity in PPP from the family members or normal controls was measured by a coupled spectrophotometric assay using  $\text{H}_2\text{O}_2$  and GSH as the cosubstrate. GSH-Px activity was significantly decreased in plasma samples from patient 1 (32%), patient 2 (60%), and their mother (56%) as compared to a normal pooled pediatric control (Fig. 7; for  $n=3$  experiments, patient 1, patient 2, and mother,  $P<0.05$  compared to control.) GSH-Px activity was not significantly decreased in plasma samples from the patients' sister or father (Fig. 7). Glutathione peroxidase activity was also measured in samples from the patients' red blood cells or platelets and found not to be significantly decreased as compared to normal controls (data not shown).

At least two isoforms of GSH-Px are selenium-dependent, the extracellular isoform and the erythrocytic isoform. To exclude selenium-deficiency as the cause of decreased GSH-Px activity, selenium levels were measured from plasma samples obtained from patients 1 and 2, their sister, parents, and individual pediatric controls. The selenium levels measured for patient 1 ( $1.18\ \mu\text{M}$ ), patient 2 ( $1.27\ \mu\text{M}$ ), and their sister ( $1.22\ \mu\text{M}$ ) were within the normal range for children ( $0.5\text{-}1.4\ \mu\text{M}$ ) and were comparable to levels measured from local pediatric controls (data not shown). Selenium levels for the patients' mother ( $1.15\ \mu\text{M}$ ) and father ( $1.27\ \mu\text{M}$ ) were within the normal range for adults ( $0.6\text{-}1.6\ \mu\text{M}$ ). In addition, other plasma antioxidant levels



were measured. Plasma levels of ascorbate, urate, and  $\alpha$ -tocopherol, as well as the low-molecular-weight thiols, glutathione, homocysteine, and cysteine were within the normal range for the brothers and mother as compared to pediatric and adult controls (data not shown).

#### **Effect of GSH-Px on Platelet Inhibition by SNO-Glu**

GFP from a normal adult donor were incubated with PPP from a family member or from a pooled pediatric control. SNO-Glu (5  $\mu$ M) was added for one minute and aggregation induced with ADP. Extent of platelet aggregation was determined in the presence or absence of GSH-Px (5 U/ml), an antioxidant enzyme that catalyzes the reduction of organic hydroperoxides and of  $H_2O_2$  (Fig. 8). The addition of GSH-Px to plasma from patient 1, patient 2, or their mother restored the platelet inhibitory response of SNO-Glu ( $P < 0.05$  for platelet incubation with GSH-Px compared to aggregation with SNO-Glu alone for each subject). When GFP were mixed with plasma from the father, sister, or pooled pediatric control, there was no significant change in aggregation after the addition of GSH-Px (Fig. 8). Platelet aggregation studies were also performed after incubating plasma from the family members or controls with SNO-Glu in the presence or absence of SOD (50 U/ml). No difference in inhibition of platelet aggregation was detected in any of these experiments (data not shown).

## DISCUSSION

In this study, a previously undescribed cause of childhood thrombotic stroke is defined in two brothers. In these patients, known causes of thrombotic strokes in childhood<sup>48</sup> were excluded, including congenital and acquired heart disease, electrolyte disturbances, thrombotic thrombocytopenic purpura, Moyamoya disease, sickle cell disease, protein C and S deficiencies, lupus anticoagulant syndrome, anticardiolipin antibody syndrome, antithrombin III deficiency, homocystinuria, and systemic lupus erythematosus.

Aggregometry and flow cytometry studies performed in whole blood showed that the brothers' platelets were hyperreactive. After exclusion of the patients' platelets as the cause of this defect, we found that, in the presence of their plasma, NO failed to inhibit aggregation or surface expression of P-selectin on normal platelets. Normal inhibition of platelet surface expression of P-selectin was seen after incubation with prostacyclin which inhibits platelet function via a cAMP-dependent pathway rather than the cGMP-dependent pathway of NO. The abnormalities observed by aggregometry and flow cytometry occurred after the addition of an exogenous NO donor (S-nitrosothiol), suggesting that alteration of the response to endogenous EDRF was not the cause of the platelet hyperreactivity and thrombosis. Therefore, the decreased plasma NO and platelet cGMP levels in these patients were probably secondary to metabolism or destruction of NO. Diluted plasma from the patients and their mother supported increased generation of  $H_2O_2$ , a reactive oxygen species that interacts with NO<sup>12</sup> and alters its physiologic effects<sup>49</sup>. GSH-Px activity was decreased in the patients' plasma as compared to control and the platelet abnormalities induced by the patients' plasma were normalized by the addition of exogenous GSH-Px, a plasma enzyme that reduces hydrogen peroxide and lipid hydroperoxides.

These data suggest that in these patients the interaction between reactive oxygen species and NO altered the normal platelet inhibitory response. *In vivo*, reactive oxygen species are formed by polymorphonuclear cells, but in our isolated system they may be formed by platelets. When stimulated by agonists, human platelets are known to produce oxidant species including

$\text{H}_2\text{O}_2$ .<sup>50</sup> In human platelets, low concentrations of  $\text{H}_2\text{O}_2$  are continuously produced with a significant rise in the concentration upon stimulation.<sup>51</sup> Hydroperoxides, such as  $\text{H}_2\text{O}_2$  and free fatty acid hydroperoxides, are formed by the stimulation of the arachidonic acid pathway in platelets leading to the lipoxygenase-dependent generation of reactive oxygen species. While superoxide is also generated by both resting and aggregating platelets, superoxide dismutase is not present in plasma<sup>52</sup> and, in our aggregation experiments, exogenous SOD failed to restore NO-induced platelet inhibition in the children. It is unlikely that the generation of reactive oxygen species directly altered the platelet response as concentrations of  $\text{H}_2\text{O}_2$  in the micromolar range are necessary to inhibit platelet aggregation.<sup>53,54</sup> The generation of reactive oxygen species also alters prostacyclin production by endothelial cells<sup>55</sup> potentially decreasing its platelet inhibitory effect but, as seen in the flow cytometry studies, NO, but not prostacyclin, failed to inhibit platelet surface expression of P-selectin in the presence of the children's plasma.

Although we measured an increase in  $\text{H}_2\text{O}_2$ , lipid hydroperoxides may also have been responsible for NO inactivation. Like  $\text{H}_2\text{O}_2$ , lipid hydroperoxides are formed during platelet aggregation and are metabolized by GSH-Px.<sup>56</sup> While not known to interact with NO directly, lipid hydroperoxides do react with hydroxyl radicals produced by aggregating platelets subsequently leading to lipid peroxidation.<sup>57</sup> Alkoxyl and peroxy radical intermediates formed during lipid peroxidation, by contrast, are known to react directly with and inactivate NO.<sup>11</sup> Hydrogen peroxide is not diffusion limited and has a half-life measured in minutes.<sup>58</sup> Therefore,  $\text{H}_2\text{O}_2$  could be metabolized by GSH-Px located in red blood cells. Conversely, lipid alkoxyl and peroxy radicals have half-lives of  $10^{-6}$  and 7 seconds,<sup>59</sup> respectively, suggesting that these lipid radicals are responsible for the inactivation of NO.

Reactive oxygen species lead to oxidative stress which can be limited by the presence of antioxidants. Plasma from the patients and their mother, capable of supporting increased  $\text{H}_2\text{O}_2$  generation, demonstrated normalization of aggregation after the addition of GSH-Px. Such a relationship has been previously suggested in patients with coronary artery disease who were

shown to have lower plasma and platelet GSH-Px levels, as well as increased platelet aggregability<sup>59</sup>. In addition, GSH-Px itself may augment platelet inhibition involving S-nitrosothiols.<sup>43</sup> While variation in plasma GSH-Px levels have been previously described in the pediatric population<sup>60</sup>, the extent of decrease reported is several-fold less than that found in the brothers' plasma. Additionally, previous reports of variability in plasma and cellular GSH-Px activity have usually been directly correlated with selenium status.<sup>61</sup> As the brothers had normal selenium levels, it is unlikely that the alteration in their GSH-Px activity was due to normal variation in this cofactor. Although enzymatic activity was not completely absent, suppression of GSH-Px activity in the clinically unaffected mother and depressed nitric oxide levels in both the mother and the sister supports a genetic component to this thrombotic process, a possibility suggested by its occurrence in the two brothers. This possibility is also supported by the normal GSH-Px levels in the brothers' and mother's platelets and red blood cells. Extracellular GSH-Px is genetically distinct from the classical cellular form and is believed to originate from hepatic<sup>62</sup> and renal cells.<sup>63</sup>

The antithrombotic effects of NO may be altered by changes in the metabolism of reactive oxygen species. In this study, we show that the plasma from two boys with a history of cerebral thrombosis can sustain increased formation of peroxide, possibly owing to a decrease in plasma GSH-Px activity. In conclusion, these data show that in these brothers, impaired metabolism of reactive oxygen species reduces the bioavailability of NO in plasma and impairs normal platelet inhibitory mechanisms. These results suggest that attenuated NO-mediated platelet inhibition produced by increased reactive oxygen species or impaired antioxidant defense may predispose to a thrombotic disorder in humans.

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**Table 1. Whole Blood Platelet Aggregometry**

	<u>Thrombin (4 U/ml)</u>	<u>Arachidonic Acid (0.6 mM)</u>	<u>Collagen (5 µg/ml)</u>
Patient 1	175	173	222
Patient 2	171	91	136
Control donors (mean of two)	107	49	32

Platelet aggregation in whole blood was induced with thrombin, arachidonic acid, or collagen, and the aggregation patterns were measured and analyzed by digitizing the area under the curve over a five minute period. Numbers represent digitized units derived from the % transmittance over 5 minutes).

## FIGURE LEGENDS

**Figure 1.** The platelets of patient 1 and, to a lesser extent, patient 2 are hyperreactive. Whole blood flow cytometry was performed on samples incubated (22°C, 10 minutes) with or without 0.25  $\mu$ M U46619 (a stable thromboxane A<sub>2</sub> analogue). The platelet surface expression of P-selectin, the glycoprotein IIb-IIIa complex, and glycoprotein Ib was detected by monoclonal antibodies S12, PAC1, and 6D1, respectively. Maximal binding of S12 and PAC1 was determined by incubation (22°C, 10 minutes) of aliquots with thrombin 2.5 U/mL. Maximal binding of 6D1 was determined by incubation (22°C, 10 minutes) of aliquots with no agonist (buffer only). (Data are mean for n = 4 control donors, and mean of 2 experiments for patients 1 and 2. Note that in the upper left and lower left panels the error bars for the controls are small and not visible)

**Figure 2.** EDRF/NO inhibits thrombin-induced platelet degranulation in the presence of control plasma, but not in the presence of plasma from patients 1 or 2. Washed platelets from normal donors were incubated (22°C, 10 minutes) with thrombin in the presence (closed circles) or absence (open circles) of 10  $\mu$ M SNAC, and in the presence of control plasma (top panel) from 3 normal donors, patient 1 plasma (middle panel), or patient 2 plasma (bottom panel). Samples were fixed and analyzed by flow cytometry with monoclonal antibody S12 (P-selectin-specific). Maximal binding of S12 was determined by incubation with 1 U/mL thrombin in the presence of control plasma and in the absence of SNAC. (Data are expressed as mean  $\pm$  SEM, n=3 experiments;  $P<0.05$  for control plasma.)

**Figure 3.** The effect of SNO-Glu on platelet aggregation. Platelets from a normal donor were incubated with PPP from family members or from a pooled pediatric control for one minute and aggregation induced with ADP. The extent of platelet aggregation was determined in the presence or absence of SNO-Glu (5  $\mu$ M). *Figure 3a.* The extent of platelet aggregation in the presence or absence of SNO-Glu using plasma from patient 2 or the (unaffected) father. *Figure 3b.* Comparison of the relative extent of platelet aggregation for experiments using PPP from the family members or from a normal pediatric control in the presence or absence of SNO-Glu

(5 $\mu$ M). For experiments conducted with PPP from the father, sister, and pooled pediatric control,  $P < 0.05$  for platelet incubation with SNO-Glu compared to aggregation with agonist alone. (Data are expressed as a percent of aggregation of a normal control with ADP alone; error bars represent SEM,  $n=3$  experiments.)

**Figure 4.** Total plasma NO concentrations. PPP from family members or from a pooled pediatric control were precipitated with perchloric acid, centrifuged, and the supernatant injected into a photolysis-chemiluminescence system to determine total NO levels. Each measurement represents the total NO (free NO and low-molecular weight S-nitrosothiols) present in the plasma sample. (Data are expressed as mean  $\pm$  SEM,  $n=3$  experiments;  $P < 0.05$  for patient 1, patient 2, sister, and mother compared to pooled pediatric control.)

**Figure 5** Platelet cGMP content. PRP from a patient, family member, or a pediatric control, was precipitated and analyzed for platelet cGMP. Concentrations of cGMP are expressed as pmol/ $10^8$  platelet. (Error bars represent SEM,  $n=3$  experiments;  $p < 0.05$  for patients 1 and 2 compared to control.)

**Figure 6.** Effect of dilute plasma on  $H_2O_2$  generation by homocysteine. The generation of  $H_2O_2$  was detected by extinction of scopoletin fluorescence during its oxidation by horseradish peroxidase over a 5-minute period in the presence of plasma (4% vol/vol) from the patient 1 (closed circle), patient 2 (closed triangle), sister (closed square), mother (open circle), father (open triangle), or a pooled pediatric control (open square). (Data are presented as the mean of two experiments for each sample point.)

**Figure 7.** Plasma glutathione peroxidase activity. The activity of GSH-Px in plasma was measured by a coupled spectrophotometric assay using  $H_2O_2$  and GSH as the cosubstrate. (For patient 1, patient 2, and mother,  $p < 0.05$  compared to control; error bars represent SEM,  $n=3$  experiments.)

**Figure 8.** The effect of GSH-Px on platelet inhibition by SNO-Glu. GFP from a normal adult donor were incubated with PPP from a family member or from a pooled pediatric control. SNO-Glu (5  $\mu$ M) was added for one minute and aggregation induced with ADP. Relative extent of

platelet aggregation was determined in the presence or absence of GSH-Px (5 U/ml). Data are expressed as a percent of control aggregation for each subject with ADP alone. (For patient 1, patient 2, and mother,  $p < 0.05$  for platelet incubation with GSH-Px compared to aggregation with SNO-Glu alone; error bars represent standard error of the mean for  $n=3$  experiments.)



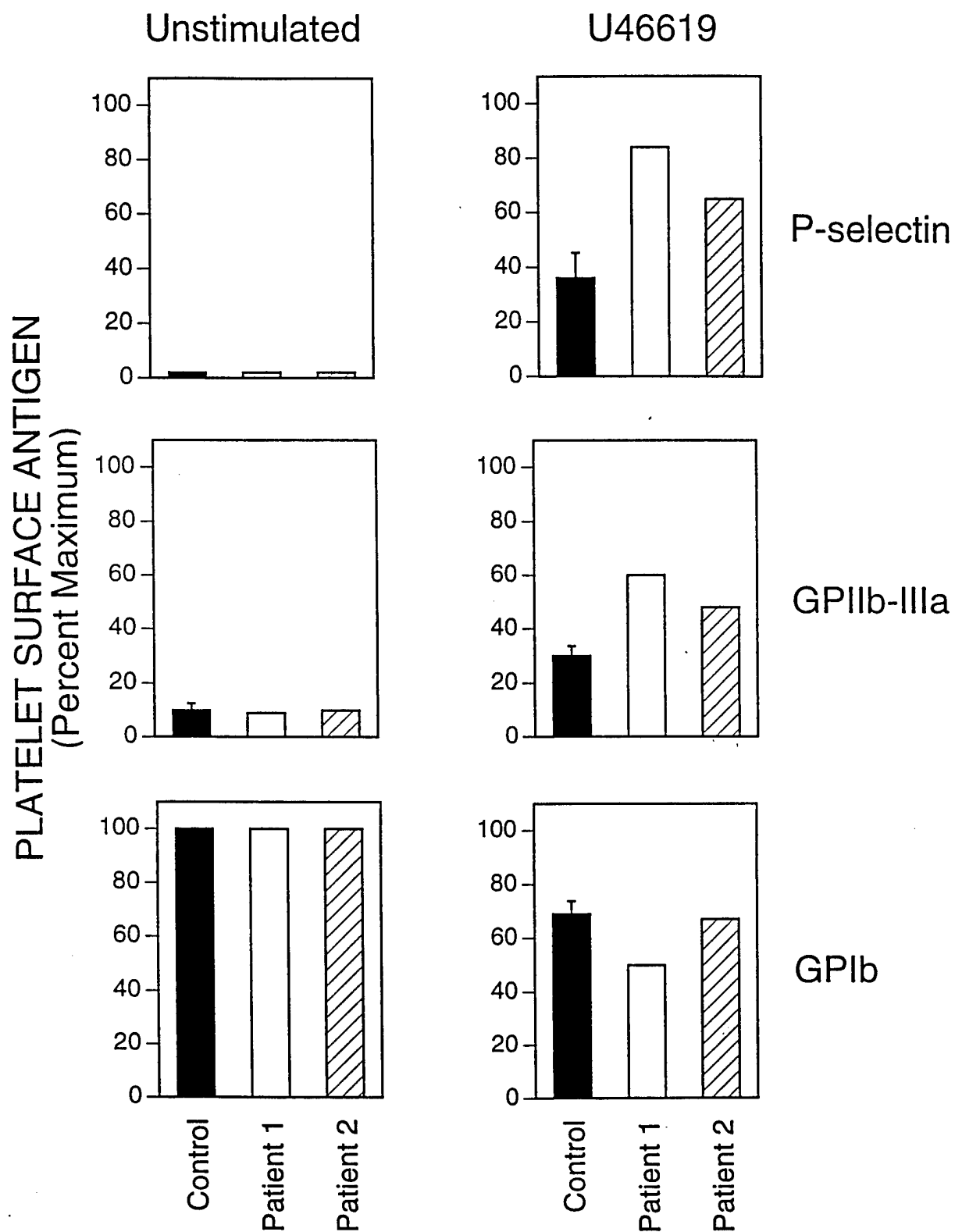


Figure 1  
Freedman et al.

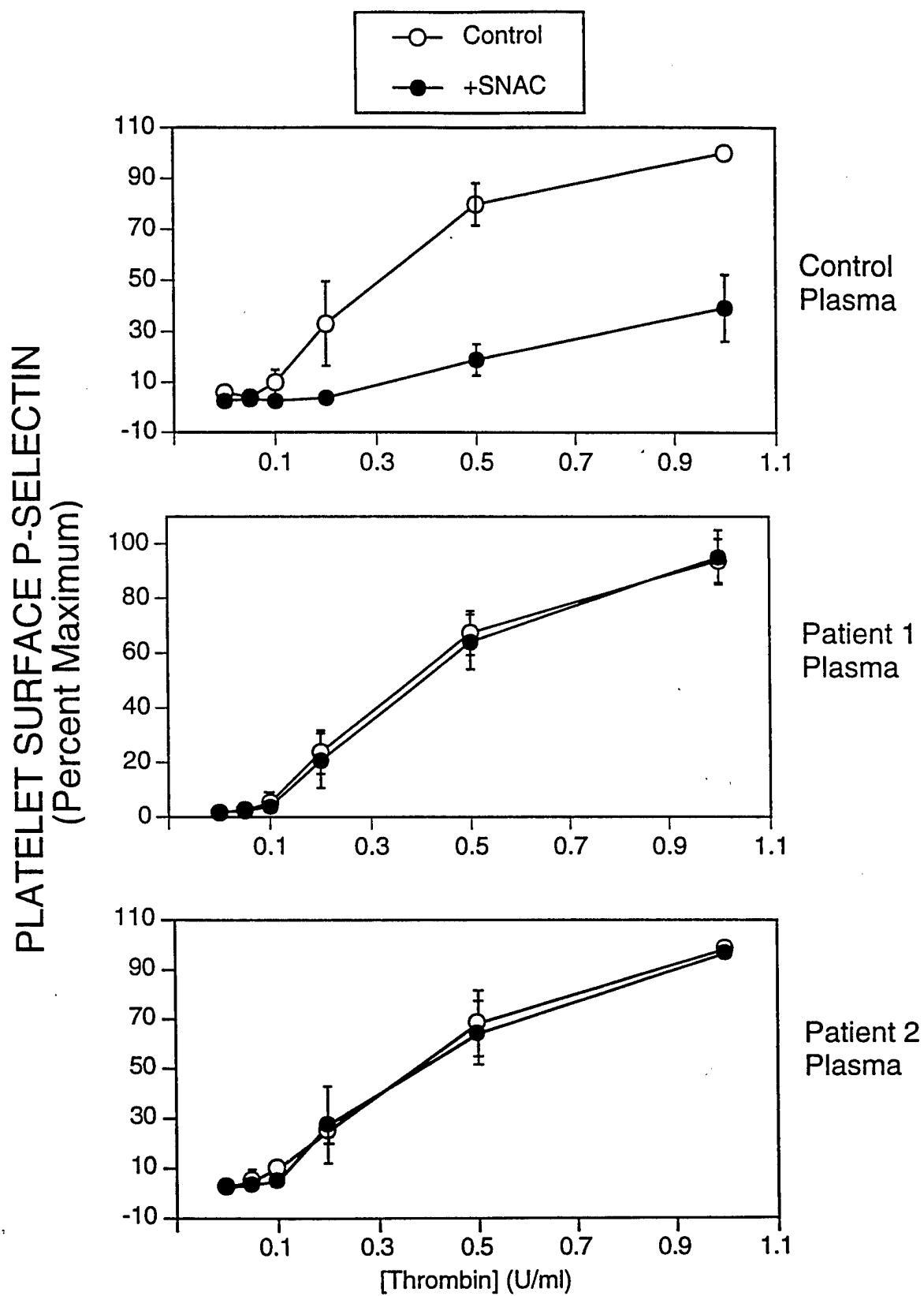


Figure 2  
Freedman et al.

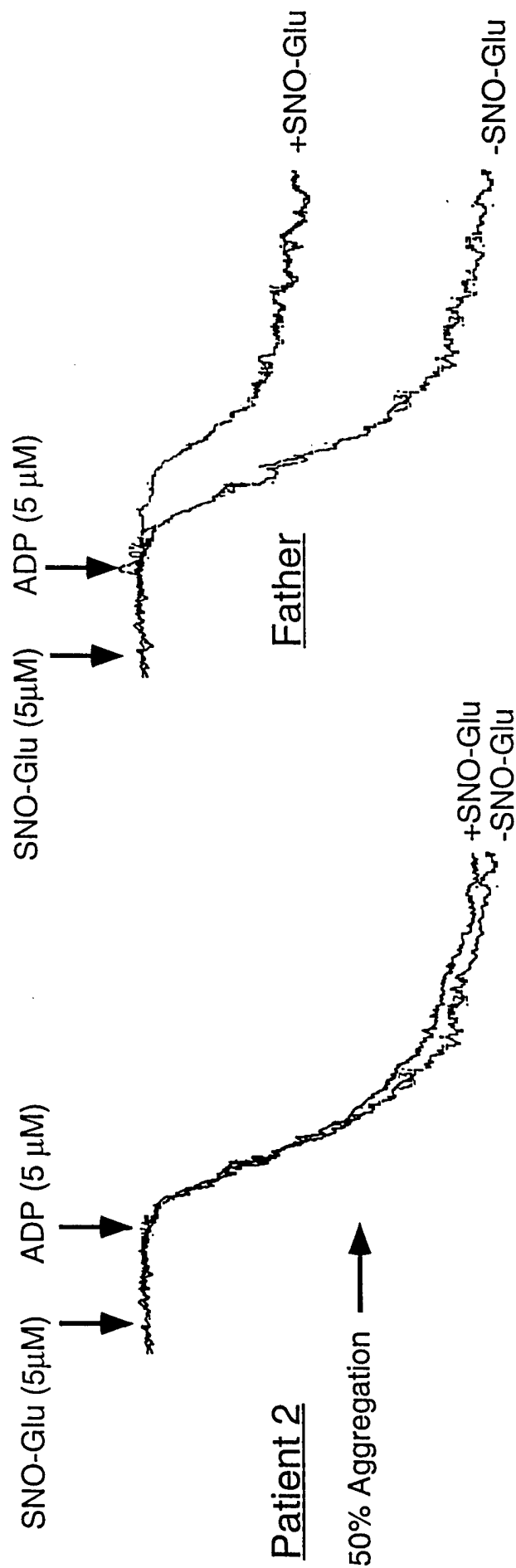


Figure 3a  
Freedman et al.

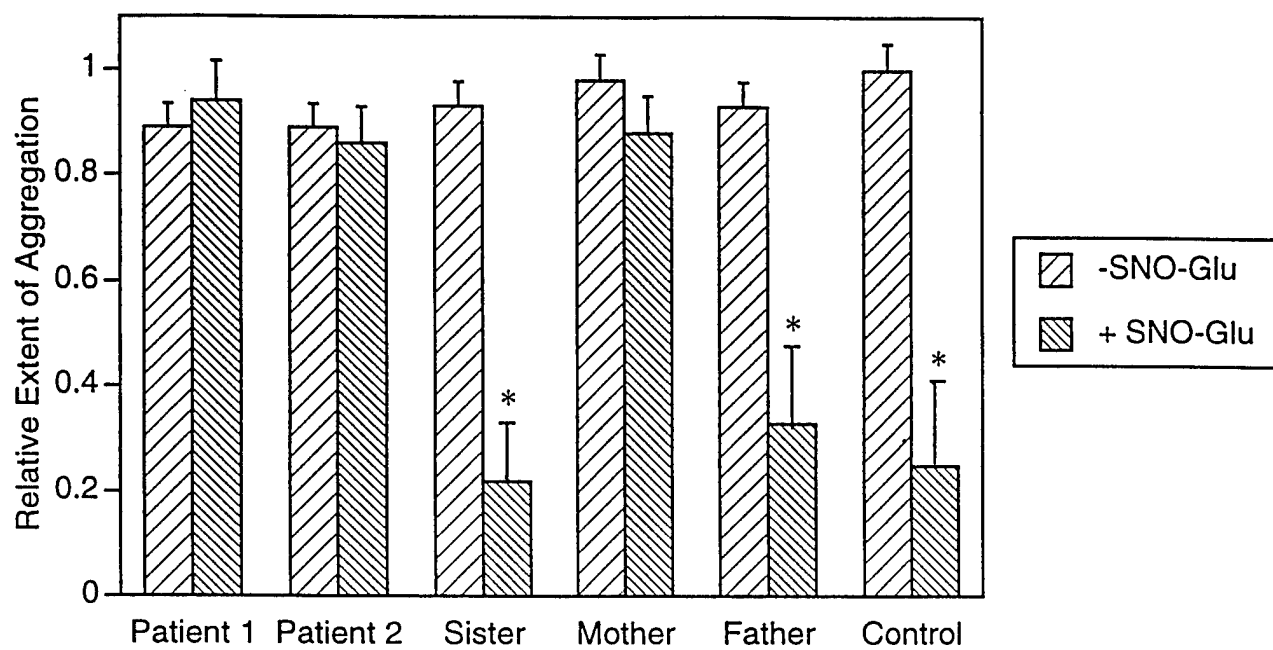


Figure 3b  
Freedman et al.

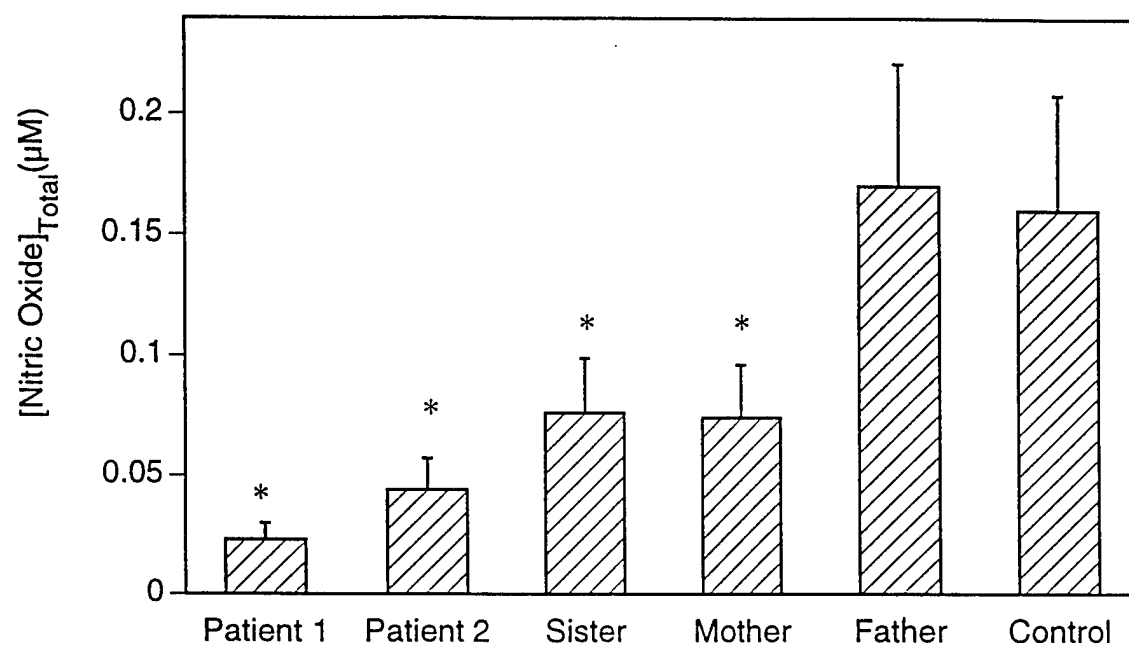


Figure 4  
Freedman et al.

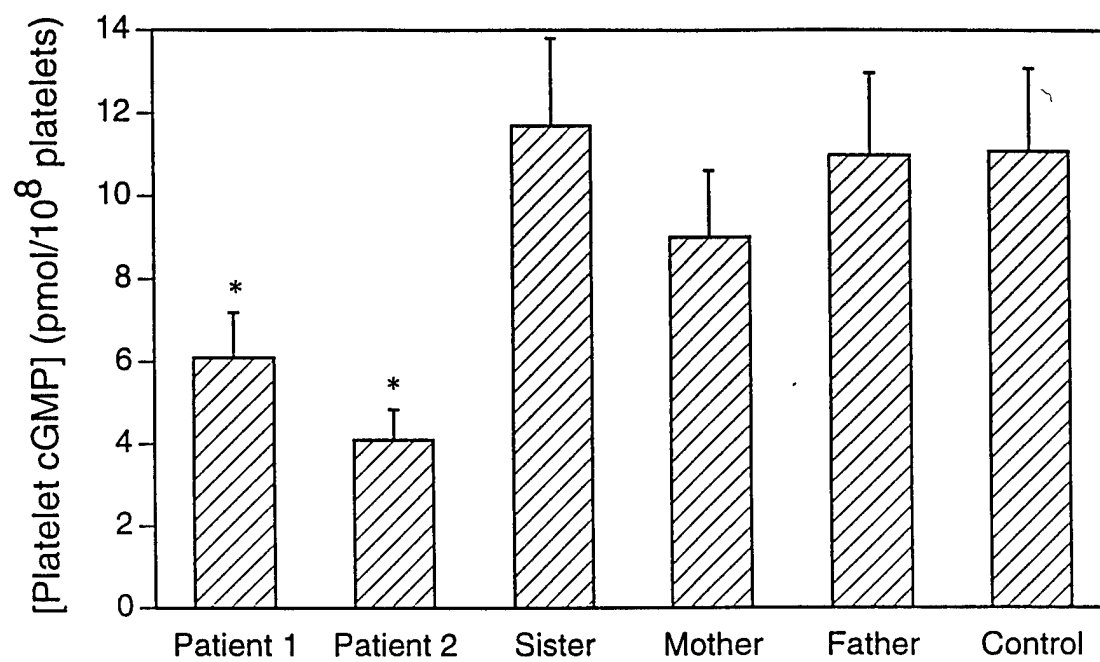


Figure 5  
Freedman et al.

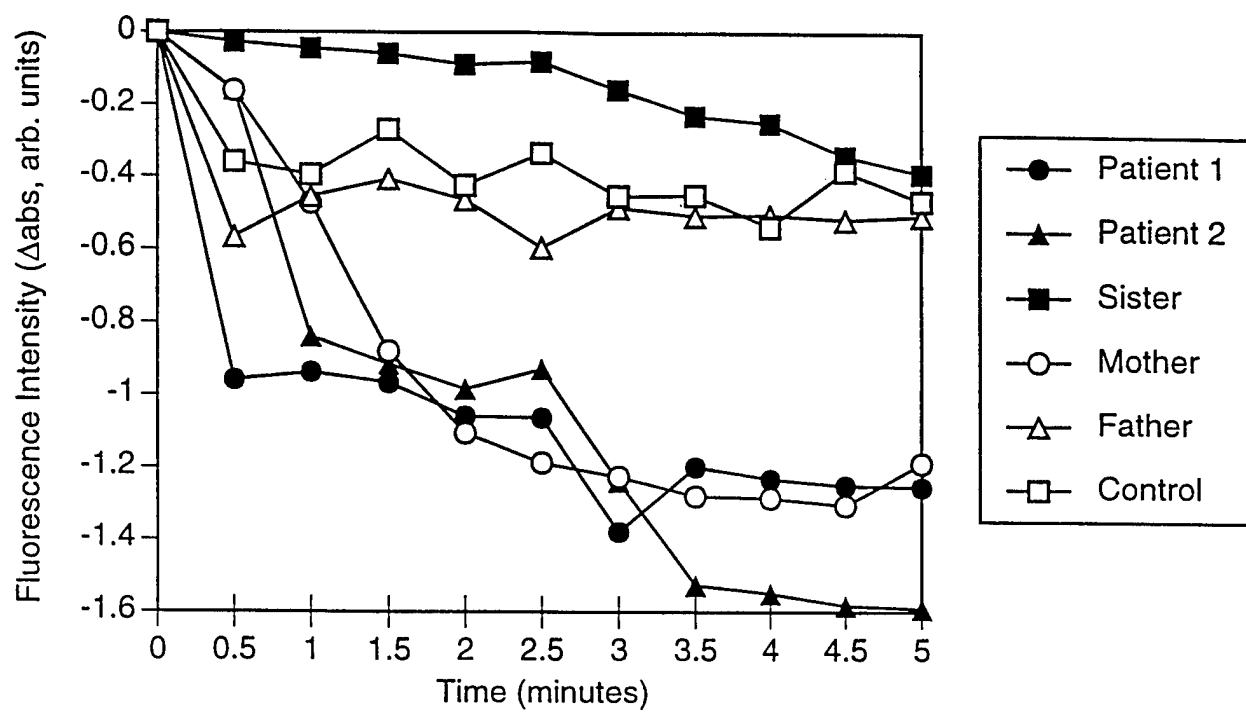


Figure 6  
Freedman et al.

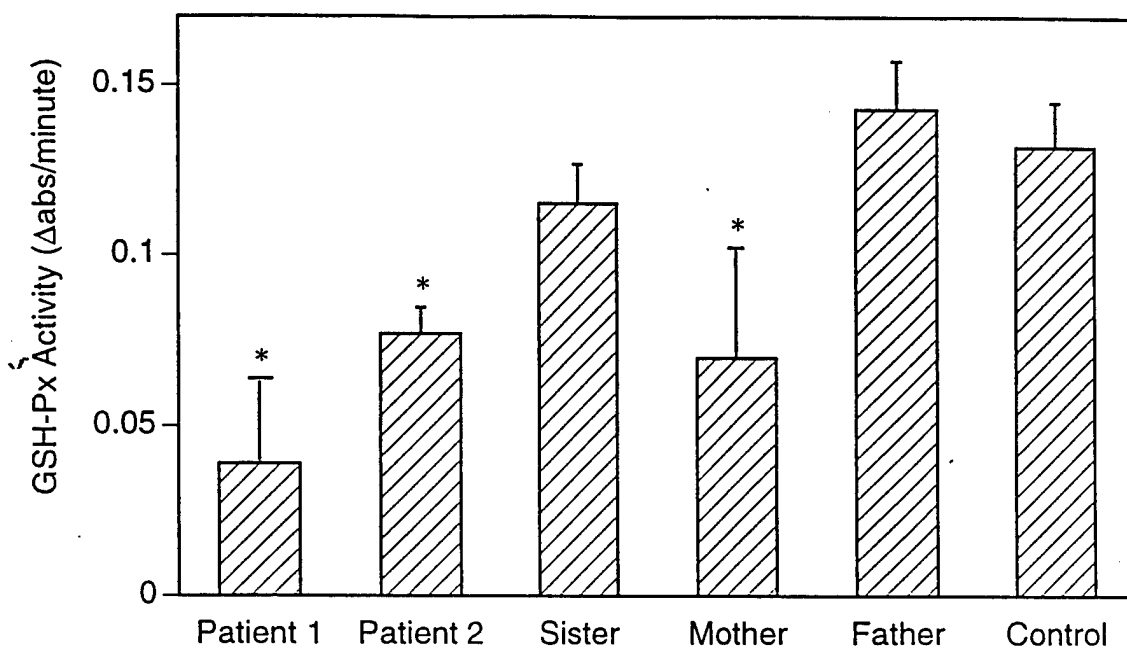


Figure 7  
Freedman et al.



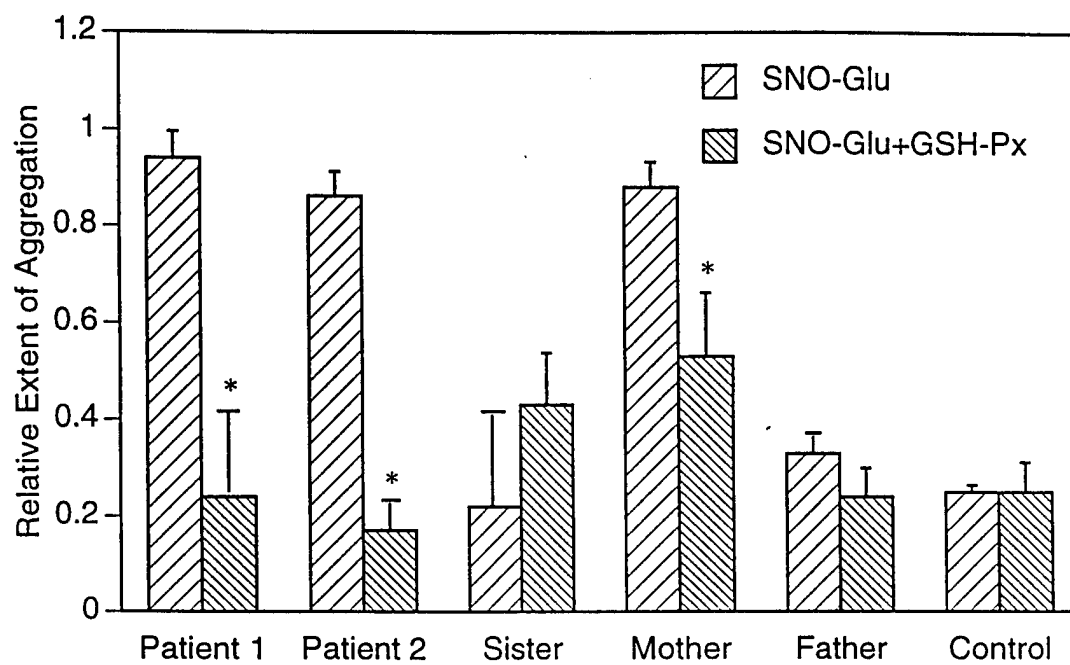


Figure 8  
Freedman et al.